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3-Keto-5 α -steroid Δ 1-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its orthologue in *Mycobacterium tuberculosis* H37Rv are highly specific enzymes that function in cholesterol catabolism

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3 **3-Keto-5 α -steroid Δ 1-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its**
4 **orthologue in *Mycobacterium tuberculosis* H37Rv are highly specific enzymes that**
5 **function in cholesterol catabolism**

6

7

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16

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19

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24 **Summary**

25

26 **The *Rhodococcus erythropolis* SQ1 *kstD3* gene was cloned, heterologously expressed**
27 **and biochemically characterized as a 3-keto-5 α -steroid Δ 1-dehydrogenase (KSTD3).**

28 **Upstream of *kstD3*, an ORF with similarity to 3-keto-5 α -steroid Δ 4-dehydrogenases**

29 **(Δ 4 KSTD) was found, tentatively designated *kst4D*. Biochemical analysis revealed**

30 **that the Δ 1 KSTD3 has a clear preference for 3-ketosteroids with a saturated A-ring,**

31 **displaying highest activity on 5 α -androstane-3,17-dione and 17 β -hydroxy-5 α -**

32 **androstane-3-one. The KSTD1 and KSTD2 enzymes on the other hand clearly**

33 **prefer (9 α -hydroxy-)4-androstene-3,17-dione as substrates. Phylogenetic analysis of**

34 **known and putative KSTD amino acid sequences showed that the *R.***

35 ***erythropolis* KSTD proteins cluster into four distinct groups. Interestingly, Δ 1**

36 **KSTD3 from *R. erythropolis* SQ1 clustered with Rv3537, the only Δ 1 KSTD present**

37 **in *Mycobacterium tuberculosis* H37Rv, a protein involved in cholesterol catabolism**

38 **and pathogenicity. The substrate range of heterologously expressed Rv3537 enzyme**

39 **was nearly identical to that of Δ 1 KSTD3, indicating that these are orthologous**

40 **enzymes. The results imply that 5 α -androstane-3,17-dione and 17 β -hydroxy-5 α -**

41 **androstane-3-one are newly identified intermediates in the cholesterol catabolic**

42 **pathway, and important steroids with respect to pathogenicity.**

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47 Introduction

48

49 3-Ketosteroid Δ^1 -dehydrogenase (KSTD) [4-ene-3-oxosteroid: (acceptor)-1-ene-
50 oxoreductase, EC 1.3.99.4] is a flavoprotein catalyzing the trans-axial elimination of the
51 C1(α) and C2(β) hydrogen atom of the A-ring from the poly-cyclic ring structure of 3-
52 ketosteroids [1]. KSTD is a key enzyme in microbial steroid catabolism needed for
53 opening of the steroid B-ring [2-5]. KSTD activities have been found in various
54 actinobacteria, e.g. *Arthrobacter* [6], *Mycobacterium* [7] and *Rhodococcus* [4, 5, 8-10], as
55 well as in *Comamonas* (formerly *Pseudomonas*) *testosteroni* [11, 12]. Choi *et al.* (1995)
56 [13] proposed to classify KSTD enzymes into two groups, based on their ability to use
57 11-substituted steroids as substrate. KSTD of *P. testosteroni* is unable to desaturate C11-
58 hydroxy- or 11-ketosteroids [14], while KSTD of *Nocardia corallina* acts on C11-
59 substituted steroids [15]. Cortisone (11-keto), but not 11 β -hydrocortisone, was found to
60 be an excellent substrate. Characterized KSTD enzymes display quite a broad substrate
61 range [7, 13, 15, 16]. Highest Δ^1 KSTD activities are observed with steroid substrates
62 carrying the 3-keto-4-ene structure: (9-hydroxy-)4-androstene-3,17-dione (4-AD) and
63 17 β -hydroxy-4-androstene-3-one (testosterone) usually are preferred substrates. Δ^1
64 KSTD activity on saturated 3-ketosteroids, e.g. 5 α -androstane-3,17-dione (5 α -AD), has
65 been observed in *P. testosteroni* [17]. However, no kinetic data for Δ^1 KSTD enzymes
66 and these saturated 3-ketosteroids is available.

67 In previous work, we have characterized the *R. erythropolis* SQ1 Δ^1 KSTD1 and Δ^1
68 KSTD2 iso-enzymes [5, 9]. Analysis of a *kstD1 kstD2* double gene deletion mutant *R.*
69 *erythropolis* strain RG8 showed stoichiometric accumulation of 9 α -hydroxy-4-

70 androstene-3,17-dione (9OHAD) from 4-AD due to blocked opening of the steroid A-
71 ring [5]. 4-AD is one of the proposed central pathway intermediates in the degradation of
72 cholesterol. However, strain RG8 is still able to degrade and grow on cholesterol as sole
73 carbon and energy source without accumulation of steroid pathway intermediates (e.g. 4-
74 AD, 9OHAD) (this study). Our subsequent studies resulted in identification of a third $\Delta 1$
75 KSTD (KSTD3) enzyme in strain RG8.

76 Analysis of the genome of the human pathogen *Mycobacterium tuberculosis* H37Rv
77 revealed the presence of a single gene (*rv3537*) encoding a putative $\Delta 1$ KSTD [4, 18, 19].
78 *M. tuberculosis* is the causative agent of tuberculosis causing millions of deaths every
79 year. The *rv3537* gene is one of several essential pathogenicity genes recently identified
80 as part of a gene cluster encoding cholesterol catabolism [19, 20]. Biochemical
81 characterization and phylogenetic analysis indicates that Rv3537 of *M. tuberculosis*
82 H37Rv is the orthologue of $\Delta 1$ KSTD3 of *R. erythropolis* SQ1 (this study). Phylogenetic
83 analysis revealed clustering of $\Delta 1$ KSTD enzymes in at least four different groups. Here
84 we report the biochemical characterization of a third and unique $\Delta 1$ KSTD (KSTD3) of *R.*
85 *erythropolis* SQ1 (and strain RG8) and its orthologue Rv3537 in *Mycobacterium*
86 *tuberculosis* H37Rv involved in cholesterol catabolism.

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93 **Experimental**

94

95 *Bacterial strains and growth conditions*

96 *Rhodococcus* strains were cultivated in complex medium at 30°C and 200 rpm. Complex
97 medium (LBP) contained 1% (wt/vol) bacto-peptone (Difco, Detroit, Mich., USA), 0.5%
98 (wt/vol) yeast extract (BBL Becton Dickinson and Company, Cockeysville, Md.) and 1%
99 (wt/vol) NaCl. *Escherichia coli* strains were grown in Luria-Bertani (LB, Sigma,
100 Zwijndrecht, The Netherlands) broth at 37°C unless stated otherwise.

101

102 *General cloning techniques*

103 Recombinant DNA techniques were done according to standard protocols [21]. *E. coli*
104 DH5 α [22] was used as general host for cloning. Transformation of *E. coli* strains was
105 done as described by Chung *et al.* (1989) [23]. DNA modifying enzymes (restriction
106 enzymes, ligase, polymerase) were purchased from Roche (Mannheim, Germany) or New
107 England Biolabs (Beverly, Mass.) and were used as described by the manufacturer.
108 Isolation of DNA restriction fragments from agarose gels was done using the Qiagen
109 (Basel, Switzerland) gel extraction kit according to protocol. DNA nucleotide sequencing
110 was done by AGOWA (Berlin, Germany). These sequence data have been submitted to
111 the DDBJ/EMBL/GenBank database under accession number EU014895.

112

113 *Preparation of Rhodococcus cell extracts and KSTD activity staining on native PAGE*

114 Overnight cultures (250 ml) of wild-type *R. erythropolis* SQ1 [24] and *kstD1 kstD2*
115 double gene deletion mutant *R. erythropolis* RG8 [5] were grown in LBP medium until

116 OD₆₆₀ of 4 and subsequently induced with steroids (1 g·L⁻¹) for an additional 4 h. Cell
117 pellets (30 min; 2500 g; 4°C) were washed with 200 ml phosphate buffer (2.72 g·L⁻¹
118 KH₂PO₄, 3.48 g·L⁻¹ K₂HPO₄, 2.46 g·L⁻¹ MgSO₄·7H₂O, pH 7.2). The washed cell
119 suspensions were concentrated to 4 ml and disrupted by passage through a French
120 pressure cell (140 Mpa) twice. Cell extracts were centrifuged for 30 min at 25000 g to
121 remove cell debris. The resulting cell free extracts were used for analysis of KSTD
122 activity on native PAGE (10% acrylamide). KSTD activity (approx. 30 -50 µg protein
123 loaded on gel) was visualized by incubating native PAGE gels in 100 ml 66.7 mM TRIS
124 buffer containing 3.1 mg phenazine methosulphate, 2.9 mg steroid (dissolved in ethanol)
125 and 41 mg nitrobluetetrazolium (NBT, dissolved in 70% dimethylformamide).

126

127 *Screening a R. erythropolis genomic library for 3-ketosteroid dehydrogenase genes*

128 Degenerate *kstD*^{deg} PCR primers [forward 5'gtgctcctggcagc(a/c)gg(g/c)gg(g/c)tt and
129 reverse 5'tgccggcggcgtacaggcct(c/t)gat] were developed based on conserved sequences
130 of amino acid sequences of Δ1 KSTD proteins and the known nucleotide sequences of
131 *kstD1* and *kstD2*, and used to screen chromosomal DNA of *R. erythropolis* RG8 for the
132 presence of novel 3-ketosteroid dehydrogenase genes. Specific *kstD*^{new} primers [forward
133 5'cttcgtgtccaatcgcgagatg and reverse 5'gacggtggcttgaaggccgg] were designed on the
134 obtained 882 bp PCR fragment from RG8 and used to screen a genomic library of *R.*
135 *erythropolis* strain RG1 in plasmid pRESQ [5] to get a clone containing the full length
136 *kstD* gene sequence. The genomic library of strain RG1 was introduced into *E. coli* DH5α
137 and plated on LB with 25 µg/ml kanamycin. All colonies on a plate were used for
138 plasmid isolation [25] and checked with PCR for positive signal with *kstD*^{new} primers.

139 PCR was performed using 30 cycles of 1 min 94°C, 1 min 60 °C and 1 min 72°C. This
140 procedure of transformation, plating dilutions and PCR was repeated several times to
141 identify a single clone, containing the pRESQ vector with a 7.4 kb insert containing
142 *kstD3*, designated pJK2 (Fig. 2).

143

144 *Heterologous expression of kstD3 in E. coli cells*

145 Total DNA of *R. erythropolis* was isolated as described [4]. PCR on total DNA was
146 performed using 30 cycles of 1 min 94°C, 1 min 60 °C and 1.5 min 72°C with forward
147 primer *kstD3*^{exp}-F [5' ggcatatgagcaagcaggaatacgcacatc] including start codon and *NdeI*
148 restriction site (underlined) and reverse primer *kstD3*^{exp}-R [5'
149 ggagatcttcaattggcatggttcgaccgcc] including stop codon and *BglIII* restriction site
150 (underlined). The resulting PCR product of *kstD3* (1,725 nt) was cloned into *NdeI/BamHI*
151 digested pET15b (Novagen) thereby introducing an N-terminal His-tag into the protein.
152 The pET15b-KSTD3 construct was introduced into *E. coli* strain BL21(DE3) (Invitrogen)
153 resulting in recombinant strain BL21pET15bKSTD3. The *kstD1* (see for PCR [4]) and
154 *kstD2* [5] genes were also cloned into the *NdeI/BamHI* site of pET15b and transformed
155 into *E. coli* BL21(DE3) resulting in recombinant strains BL21pET15bKSTD1 and
156 BL21pET15bKSTD2.

157 Hundred-fold diluted pre-cultures of recombinant *E. coli* strains were grown for 48 h at
158 16 °C, 200 rpm, in LB broth supplemented with 0.5 M sorbitol and ampicillin (100
159 µg/ml). Isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration
160 of 0.1 mM at inoculation (*kstD1*, *kstD2* expression) or after 24 h of incubation for *kstD3*
161 expression. After 24 h (*kstD3*) or 48 h (*kstD1*, *kstD2*) induction, cells were harvested by

162 centrifugation (2600 g, 10 min) and dissolved in phosphate buffer (2.72 g·L⁻¹ KH₂PO₄,
163 3.48 g·L⁻¹ K₂HPO₄, 2.46 g·L⁻¹ MgSO₄·7H₂O, pH 7.2). Cells were disrupted by French
164 press and cell debris was precipitated by centrifugation at 25000 g for 30 min.
165 Supernatants were used for kinetic studies with a range of substrates.

166

167 *Identification of steroids by HPLC*

168 Cell free extract of *E. coli* expressing KSTD3 (50-200 µg protein) was incubated with
169 200 µM 2,6-dichlorophenolindophenol (DCPIP), 200 µM 5α-AD in 50 mM Tris buffer
170 (pH 7.4) for 1 h at 30 °C in a total volume of 0.5 ml. Subsequently, 2 ml of 80% methanol
171 was added and the sample was incubated for 10 min at room temperature and analyzed by
172 HPLC.

173 Steroids were separated on an Alltima C18 column (250 mm x 4.6 mm, 5 µm) at 35°C,
174 using a mobile phase consisting of MeOH:H₂O (80:20), and detected at a wavelength of
175 254 nm.

176

177 *KSTD enzyme activity standard assay*

178 The kinetics of the KSTD1, KSTD2 and KSTD3 enzymes were determined by incubating
179 the respective cell free extracts with 5α-androstane-3,17-dione (5α-AD) (Steraloids,
180 Newport, Rhode Island, USA), 17β-hydroxy-5α-androstane-3-one (5α-T) (Sigma,
181 Zwijndrecht, The Netherlands), 4-androstene-3,17-dione (4-AD) (Organon Biosciences,
182 Oss, The Netherlands), 9α-hydroxy-4-androstene-3,17-dione (9OHAD) (Organon
183 Biosciences), progesterone (P) (ICN Biomedicals Inc., Hyland Ave, Costa Mesa, USA),
184 11β-hydrocortisone (Sigma), 23,24, bisnor 5α-cholestane-3-one acid (Steraloids), 5β-

185 androstane-3,17-dione (Steraloids), 1-(5 α)-androstene-3,17-dione (Steraloids) (1-(5 α -
186 AD), 11 α -hydroxy-4-norandrostene-3,17-dione (Organon Biosciences), 1,4-
187 androstadiene-3,17-dione (ADD) (Organon Biosciences), dehydroisoandrosterone
188 (Organon Biosciences), 5 α -pregnan-3,20-dione (5 α -P) (Steraloids), 4-cholesten-3-one
189 (Sigma), 5 α -cholestan-3-one (Acros organics, Geel, Belgium) and 5 α -androstane-3 β -ol,
190 17-one (Steraloids). Enzyme activities were measured spectrophotometrically at 30 °C
191 using DCPIP (extinction coefficient of 21 mM⁻¹cm⁻¹) as artificial electron acceptor.
192 OD₆₀₀-measurements were followed with the “Soft-max PRO 4 (lifescience edition)”
193 program. Curve fitting of the data was performed with the “SigmaPlot” program (version
194 10) using the Michaelis-Menten formula $[y=(a * x)/(b + x)]$.
195 The reaction mixture of 1 ml consisted of 50 mM TRIS pH 7.4, 80 μ M DCPIP, *E. coli*
196 cell-free extracts (see above) and varying concentrations of steroid substrates dissolved in
197 ethanol (or methanol in case of 5 α -pregnan-3,20-dione and 5 α -cholestan-3-one). No
198 activity was detected in control reaction mixtures lacking steroids or cell-free extracts, or
199 extracts of *E. coli* with empty pET15b expression vector.

200

201 *Phylogenetic tree construction*

202 Phylogenetic and molecular evolutionary analyses were conducted using MEGA version
203 3.1 [26]. Amino acid sequences of different KSTD enzymes of different actinomycetes
204 and of *Comamonas testosteroni* were obtained from Genbank or TIGR. The amino acid
205 sequences (accession number between brackets) of KSTD1 (AAF19054), KSTD2
206 (AAL82579), KSTD3 (ABW74859) and KSTD4 (ABW74858) of *R. erythropolis* SQ1,
207 KsdD (AB007847) of *Rhodococcus rhodochrous*, KsdD (BAA07186) of *Arthrobacter*

208 *simplex*, MAP0530 (NP959464), MAP1433c (NP960367), MAP3398c (NP962332),
209 MAP0696 (NP959670), MAP1530 (NP960464) and MAP1447c (NP960381) of
210 *Mycobacterium avium subsp. paratuberculosis str. k10*, MSMEG5941 (YP890167),
211 MSMEG2869 (YP887189), MSMEG4864 (YP889120), MSMEG2867 (YP887187),
212 MSMEG4870 (YP889125), MSMEG2873 (YP887193) and MSMEG3657 (YP887960)
213 of *Mycobacterium smegmatis*, Rv3537 (CAB05041) and Rv1817 (CAB01462) of
214 *Mycobacterium tuberculosis* H37Rv, nfa4630 (BAD55305), nfa40910 (BAD58940),
215 nfa22440 (BAD57091), nfa4640 (BAD55306) and nfa30830 (BAD57930) of *Nocardia*
216 *farcinica* IFM 10152, ro05798 (YP705733), ro02483 (YP702446), ro04532 (YP704476),
217 ro09040 (YP708242), ro09023 (YP708225), ro05813 (705748) and ro05698 (YP705635)
218 of *Rhodococcus sp.* RHA1, TesI (BAC81693) and TesH (BAC81692) of *Comamonas*
219 *testosteroni* and orf00954, orf01263, orf01472, orf01880, orf04377 and orf06377 (orf
220 Glimmer prediction) of *Rhodococcus equi* were used for phylogenetic tree construction.
221 The sequence data of *Rhodococcus equi* were produced by the *Rhodococcus equi*
222 Sequencing Group at the Sanger Institute and were obtained from
223 <ftp://ftp.sanger.ac.uk/pub/pathogens/re>.
224
225 *Heterologous expression of Rv3537 of Mycobacterium tuberculosis H37Rv in E. coli cells*
226 PCR was performed on genomic DNA of *M. tuberculosis* H37Rv (Dick van Soolingen,
227 RIVM, The Netherlands) using 30 cycles of 1 min 94°C, 1 min 60 °C and 1.5 min 72°C,
228 with forward primer *kstD*^{Rv3537}-F [5' cgctcgagatgactgtgcaggagttcgacg] including start
229 codon and *Xho*I restriction site (underlined) and reverse primer *kstD*^{Rv3537}-R [5'
230 cgagatcttcagcgcttccccgcctgac] including stop codon and *Bgl*/II restriction site (underlined).

231 The PCR product was ligated into the *XhoI/BamHI* restriction site of the pET15b-vector.
232 The pET15b-Rv3537 construct was transformed into *E. coli* strain BL21(DE3) resulting
233 in strain BL21pET15bRv3537. The protein KSTD3_{H37Rv} was expressed under the same
234 conditions as KSTD3_{SQ1}.

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235 **Results and discussion**

236

237 *Identification of additional KSTD activities in *kstD1 kstD2* mutant RG8*

238 Deletion of the 3-ketosteroid- Δ 1-dehydrogenase genes *kstD1* and *kstD2* in *R. erythropolis*

239 SQ1 did not result in accumulation of steroid intermediates during incubation of strain

240 RG8 with cholesterol [5]. We now also observed that strain RG8 still grows normally on

241 cholesterol as sole carbon source. Therefore, we predicted the presence of additional

242 KSTD enzymes in *R. erythropolis* SQ1. KSTD activity staining, first with 4-AD (a Δ 1

243 KSTD substrate) and subsequently with 1-(5 α)-androstene-3,17-dione (1-5 α -AD) (a Δ 4

244 KSTD substrate) visualized activity bands for two Δ 1 KSTD enzymes and for one Δ 4

245 KSTD enzyme in wild-type *R. erythropolis* SQ1 (Fig. 1). Only KSTD1 enzyme activity

246 band is missing in the *kstD1 kstD2* mutant strain *R. erythropolis* RG8 (Fig. 1) (see also

247 [5]). The Δ 4 KSTD and second Δ 1 KSTD enzyme have not been characterized yet. The

248 steroids progesterone (P), 4-AD and 9OHAD each induce two Δ 1 KSTD enzymes and

249 one Δ 4 KSTD enzyme, although these inducing compounds are substrates for Δ 1 KSTD

250 enzymes.

251 Figure 1 thus demonstrates the natural diversity of 3-ketosteroid-dehydrogenases in *R.*

252 *erythropolis*. The number of KSTD activities shown in Fig. 1 is even an underestimation,

253 since some KSTD enzymes (for instance KSTD2) can not use NBT as electron acceptor

254 and are not detected in this assay [5].

255 The two KSTD activities observed in *kstD1 kstD2* mutant strain RG8 (Fig. 1) indicate

256 that, in addition to KSTD1 and KSTD2, *R. erythropolis* harbors at least one more Δ 1

257 KSTD and at least one Δ 4 KSTD activity. In subsequent work we have cloned and

258 characterized additional *kstD* genes that might be responsible for the ongoing
259 consumption of cholesterol in the *kstD1 kstD2* mutant strain RG8.
260
261 *Isolation and cloning of additional kstD genes from Rhodococcus erythropolis SQ1*
262 Alignment of annotated (e.g. KSTD1, KSTD2) and putative $\Delta 1$ KSTD protein sequences
263 identified by similarity searches in databases (<http://www.ncbi.nlm.nih.gov/BLAST/>)
264 revealed the presence of conserved domains. Based on two conserved domains, the
265 VVLAAGGF motif (a.a. 225-232 in KSTD1_{SQ1}) and GLYAAG motif (a.a. 471-476 in
266 KSTD1_{SQ1}), a set of degenerate PCR primers was designed and used for screening strain
267 RG8 for the presence of additional *kstD* sequences. A PCR product of approximately 0.9
268 kb was obtained and sequenced. The deduced amino acid sequence was similar, but not
269 identical, to KSTD1 and KSTD2 from *R. erythropolis* SQ1, suggesting that a third *kstD*
270 gene had been cloned. The full length gene was subsequently isolated by screening a
271 genomic library of *R. erythropolis* RG1 [5] with specific PCR primers based on the
272 nucleotide sequence of the 0.9 kb PCR product. A positive clone (pJK2), carrying a 7.4
273 kb insert (GC-content 63.3%), was identified and sequenced. Bioinformatic analysis of
274 the nucleotide sequence revealed a total of 6 ORFs, two of which showed similarity to
275 *kstD* genes (Fig. 2). One ORF (*kst4D*) encodes a protein displaying high similarity (45%
276 identity) with 3-ketosteroid- $\Delta 4(5\alpha)$ -dehydrogenase of *Comamonas testosteroni* TA441
277 [12], while the second, divergently transcribed ORF (1,725 nt, GC content 63.6%, *kstD3*)
278 encodes a protein with high similarity to $\Delta 1$ KSTD1 (37% identity) and KSTD2 (42%
279 identity) of *R. erythropolis* SQ1 (Fig. 2; Table 1; [4], [5]). KSTD3 displays highest
280 similarity (78% identity) with Ro04532 of *Rhodococcus* sp. RHA1. Transcriptomic

281 analysis recently showed that *ro04532* is upregulated during growth on cholesterol, thus
282 probably catalyzing a $\Delta 1$ -dehydrogenation reaction during cholesterol degradation [19].
283 Four additional ORFs were identified on the cloned DNA fragment, and all appear to be
284 involved in cholesterol catabolism (Fig. 2; Table 1; [19]). The *choG* gene encodes a
285 protein highly similar (72%) to cholesterol oxidase from *Brevibacterium sterolicum* [27].
286 Homologues of *hsaE* and *hsaG* have been identified in *Rhodococcus* sp. RHA1 and were
287 predicted to function in cholesterol ring degradation (Table 1; [19]). The gene *hsd4B* is
288 homologous to part of the eukaryotic gene encoding the central domain of 17 β HSD4, and
289 to the *hsd4B* gene of *Rhodococcus* sp. RHA1, which is a 2-enoyl acyl-CoA hydratase,
290 proposed to catalyze a step in the cholesterol side-chain shortening (Table 1; [19])
291 The genetic organization of this cluster is (semi)conserved in several other actinomycetes,
292 e.g. in *Nocardia farcinica*, *Rhodococcus* sp. RHA1 and *Mycobacterium tuberculosis* [10,
293 18, 28], indicating a highly relevant role in cholesterol catabolism.

294

295 *Heterologous expression of kstD3 in E. coli*

296 The *kstD3* gene of strain *R. erythropolis* SQ1 was heterologously expressed in *E. coli*
297 strain BL21(DE3) for further biochemical analysis. *E. coli* strain BL21(DE3), containing
298 the pET15b-KSTD3 expressed active KSTD3 at moderate levels (approx. 5% of total
299 protein, every lane contains approx. 50 μ g protein, Fig. 3) when grown for 2 days at 16
300 $^{\circ}$ C. The *kstD3* gene encodes a protein of 574 amino acids with a calculated molecular
301 weight of 61 kDa, which corresponds to the band of KSTD3 on the SDS PAGE gel (Fig.
302 3). The expression of KSTD3 is minimal compared to expression of KSTD1 (approx.
303 50% of total protein) and KSTD2 (approx. 20% of total protein). Expression at higher

304 temperatures resulted in production of inactive KSTD3. Attempts to purify active His-
305 tagged KSTD3 protein were unsuccessful, since KSTD3 activity was lost upon
306 purification using a Ni-NTA column. Despite exhaustive attempts, using different elution
307 agents (e.g. NaCl, Imidazol, histidine) or additives (e.g. DTT, FAD, FMN, glycerol), we
308 were unable to obtain purified active KSTD3 protein. Since the *E. coli* background lacks
309 all KSTD activity, we decided to perform subsequent biochemical analysis of KSTD3
310 with cell-free extracts.

311

312 *KSTD3 displays 3-keto-5 α -steroid Δ 1-dehydrogenase activity*

313 Based on sequence similarities with other Δ 1 KSTD proteins, KSTD3 is expected to
314 encode Δ 1 KSTD activity. However, incubations of cell free extracts containing KSTD3
315 with 4-AD (200 μ M) did not reveal detectable initial activity in a standard assay using
316 DCPIP as artificial electron acceptor. Interestingly, Δ 1 KSTD enzymes characterized thus
317 far all display relatively high activities with 4-AD [1, 4, 5, 7, 13, 16]. A range of steroid
318 substrates was subsequently tested with KSTD3 (Table 2) and activity was detected with
319 5 α -AD. To verify that KSTD3 indeed performs Δ 1-dehydrogenation of 5 α -AD, product
320 formation in a standard assay was analyzed by HPLC. Incubation of KSTD3 with 5 α -AD
321 resulted in the formation of 1-(5 α)-AD, the expected product of 5 α -AD Δ 1-
322 dehydrogenation, identified using authentic 1-(5 α)-AD.

323 Interestingly, HPLC analysis also revealed that KSTD3 in *E. coli* cell-free extracts
324 catalyzes Δ 1-dehydrogenation of 5 α -AD in the absence of an artificial electron acceptor,
325 suggesting that KSTD3 uses O₂ as electron acceptor. Oxidase activity has been
326 previously described for KSTD of *Nocardia corallina* [15]. Intriguingly, aerobic

327 dehydration by KSTD has been linked to transmembrane potential generation in
328 *Arthrobacter globiformis* [29]. KSTD3 thus may have a similar function in *Rhodococcus*.
329
330 *KSTD3 displays a narrow substrate specificity compared to KSTD1 and KSTD2*
331 Cell free extracts of *E. coli* BL21(DE3), containing either KSTD1, KSTD2 or KSTD3,
332 were used to compare the substrate specificity of KSTD3 with that of KSTD1 and
333 KSTD2 (Table 2). KSTD activity was not observed in control cell-free extracts of *E. coli*
334 BL21(DE3) harboring the expression vector without a *kstD* gene. Clearly, KSTD3 has a
335 very narrow substrate range compared to KSTD1 and KSTD2. KSTD3 activity was
336 detected with 5 α -AD, 17 β -hydroxy-5 α -androstane-3-one (5 α -testosterone, 5 α -T) and 5 α -
337 pregnane-3,20-dione (5 α -P), but not with 5 β -H or 3-hydroxy isomers tested. KSTD
338 enzymes require a carbonyl oxygen at C3 that interacts with an electrophilic residue,
339 thereby facilitating the extraction of the C2(β) hydrogen as a proton by a nucleophilic
340 residue [30]. It is thought that an essential histidine is acting as a nucleophile [31].
341 5 α -T appears to be the preferred KSTD3 substrate, with an approximately 2-fold lower
342 K_{Mapp} compared to 5 α -AD. A 5 α -H-3-keto configuration of the steroid substrate appears
343 to be important for high KSTD3 activity. KSTD3 activity was not observed with 5 α -
344 steroids having a bulky side chain at C-17, indicating that only steroids carrying a small
345 (5 α -P), or no aliphatic side chain (5 α -AD, 5 α -T) are suitable substrates for KSTD3.
346 Contrary to KSTD1 and KSTD2, KSTD3 does not, or hardly at all, display activity with
347 the common unsaturated Δ^4 steroids (e.g. 4-AD, 9OHAD), with the exception of a minor
348 activity observed for 4-pregnene-3,20-dione (progesterone). The 5 α -3-ketosteroids 5 α -
349 AD and 5 α -T are substrates for all three Δ^1 KSTD enzymes studied. KSTD3 can be

350 distinguished from KSTD1 and KSTD2 by its preference for steroids with a saturated A-
351 ring. KSTD3 and KSTD2 both can not use NBT as an artificial electron acceptor; the
352 unknown $\Delta 1$ KSTD activity band in Figure 1 is therefore not the KSTD3 protein
353 characterized in this report.

354 The genomic co-location of *kstD3* with other cholesterol catabolic genes in *R.*
355 *erythropolis* SQ1 (Table 1; Fig. 2), as well as strong up-regulation of its homologue in
356 cholesterol grown cells of *Rhodococcus* sp. RHA1 [19], indicate a role for KSTD3 in
357 cholesterol degradation. Unexpectedly, the preferred substrate of KSTD3 is not 4-AD, the
358 commonly accepted central intermediate in sterol/steroid degradation, but the 5α -reduced
359 form of 4-AD. The data thus suggest that 5α -T and 5α -AD are pathway intermediates
360 preceding 1,4-androstadiene-3,17-dione (ADD) during cholesterol catabolism. The
361 conversion of 5α -AD into ADD requires the action of a $\Delta 1$ KSTD, encoded by *kstD3*,
362 and a $\Delta 4$ KSTD, putatively encoded by *kst4D*. Both activities appear to be encoded by the
363 cloned DNA fragment (Table 1; Fig. 2).

364

365 *Phylogeny of (putative) 3-ketosteroid dehydrogenases of several actinomycetes*

366 The genome sequences of several actinomycetes were analyzed for KSTD homologues
367 by searching for high hits with the amino acid sequences of KSTD1, KSTD2, KSTD3
368 and $\Delta 4$ KSTD. Construction of a phylogenetic tree, based on the complete amino acid
369 sequences of the different (putative) KSTDs, revealed that the $\Delta 1$ KSTDs cluster into at
370 least four distinct groups (Fig. 4). Three of the four groups also have a $\Delta 1$ KSTD
371 representative of *R. erythropolis*. *Mycobacterium smegmatis* has 6 putative $\Delta 1$ KSTDs
372 [32], one of which does not belong to any of the groups in this phylogenetic tree (Fig. 4).

373 This suggests that the natural diversity of $\Delta 1$ KSTDs is even more elaborate than 4
374 groups. Among the $\Delta 1$ KSTDs, the identity of the KSTD3 amino acid sequences are
375 slightly more conserved (64-73%) as compared to KSTD1 (40-70%) and KSTD2 (58-
376 70%). The KSTD enzymes display significant similarity with fumarate reductases (Frd;
377 20-24%). Alignment of amino acid sequences of Frd of *Shewanella putrefaciens* with
378 those of $\Delta 1$ KSTDs revealed four conserved regions (I-IV; Fig. 5) in addition to the
379 conserved FAD-binding domain in the N-terminal part of the protein [6, 17]. Region I
380 has been suggested to comprise the active site residues of KsdD of *A. simplex*, based on
381 an alignment with the active site of Frd [6]. However, our alignment indicates that the
382 active site residues H365, E378 and R381 of Frd do not align with Region I, as was
383 proposed for KsdD of *A. simplex*, but are scattered around conserved Region II. Active
384 site residue R402 of Frd, responsible for donation of a proton to the substrate [33-36], is
385 also found near region II and interestingly is occupied by a tyrosine in most KSTDs (Fig.
386 5). Tyrosine residues have been implicated as important for KSTD activity [37]. Residues
387 Y104 and Y116 in KsdD of *R. rhodochrous*, thought to be involved in steroid substrate
388 binding, however, are not conserved in the different types of $\Delta 1$ KSTDs. Residue Y121
389 in KsdD of *R. rhodochrous*, on the other hand, is conserved among all $\Delta 1$ KSTDs and
390 was shown to be essential for catalysis [37]. Conserved region IV includes two key
391 substrate-binding residues identified in Frd, i.e. H504 and R544 [33-36]. Residue H505 in
392 region IV may be involved in modulation of the pK_a of H504, while residues T506,
393 M507, G508, E534 and T536 in Frd are important for coordinating a sodium ion
394 positioned close to both the FAD-cofactor and the active site of Frd [38]. The threonine
395 residue, corresponding to T506 of Frd in region IV, is fully conserved among KSTD

396 enzymes and was suggested to be important for substrate binding or catalysis in KSTD2
397 of *R. erythropolis* SQ1 [5]. Interestingly, the cysteine residue located next to this
398 conserved threonine seems to be characteristic for KSTD2 enzymes.

399

400 *Rv3537* of *M. tuberculosis* H37Rv is a KSTD3 orthologue

401 Remarkably, among the $\Delta 1$ KSTDs only the KSTD3 clusters with a putative KSTD of *M.*
402 *tuberculosis* (Fig. 4). The putative 3-ketosteroid dehydrogenase *Rv3537* of *M.*

403 *tuberculosis* H37Rv is essential for survival in the macrophage [20]. To analyze whether

404 the biochemical characteristics of different KSTDs in one distinct cluster are similar, the

405 substrate profiles of *Rv3537* of *M. tuberculosis* and KSTD3 of *R. erythropolis* were

406 compared. *Rv3537* from *M. tuberculosis* H37Rv was expressed in *E. coli* BL21(DE3),

407 using the same conditions as for KSTD3 expression. $\Delta 1$ KSTD enzyme assays and HPLC

408 product analysis revealed a highly similar substrate range and apparent substrate affinities

409 for *Rv3537* compared to KSTD3 (Table 2). The substrate range for *Rv3537* was even

410 more limited than for KSTD3, since its $\Delta 1$ KSTD activity towards 5α -P was below

411 detection limits.

412 Similarity searches and phylogenetic analysis show that *Rv3537* of *M. tuberculosis*

413 H37Rv is the only $\Delta 1$ KSTD enzyme present in H37Rv [18]. *Rv3537* plays an important

414 role in macrophage survival of *M. tuberculosis* [20] and in the cholesterol catabolic

415 pathway [19], suggesting that 5α -AD and/or 5α -T are important steroid intermediates

416 with respect to pathogenicity. KSTD exclusively occurs as a bacterial enzyme, i.e. there

417 are no known human orthologues, marking KSTD as an interesting new drug target to

418 fight tuberculosis.

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560 **Figure legends**

561

562 **Figure 1 KSTD activity staining in *Rhodococcus erythropolis* SQ1**

563 KSTD activity was visualized by adding first 4-androstene-3,17-dione (4-AD) and
564 subsequently 1-(5 α)-androstene-3,17-dione (1-(5 α)-AD) as substrates, on native PAGE
565 gel loaded with cell free extracts (30-50 μ g of protein) of wild type *R. erythropolis* SQ1
566 (WT) and *kstD1kstD2* gene deletion mutant RG8 (RG8) induced with 4-AD, 9 α -hydroxy-
567 4-androstene-3,17-dione (9OHAD) or progesterone (P), revealing activity bands of Δ 1 3-
568 ketosteroid dehydrogenases (Δ 1 KSTD) (the lower two bands, which appear after adding
569 4-AD) and a Δ 4 3-ketosteroid dehydrogenase (Δ 4 KSTD) (the upper band, which appears
570 after adding 1-(5 α)-AD). Although two Δ 1 KSTD encoding genes have been knocked out
571 in mutant strain RG8, only one activity band is disappearing (KSTD1, [4]).

572

573 **Figure 2 Gene cluster and their proposed role in cholesterol degradation**

574 DNA fragment of *R. erythropolis* SQ1 (7.4 kb) containing sterol/steroid degradation
575 genes (Table 1). Black arrows represent complete genes and open arrows incomplete
576 genes. Enzymes between brackets are not located on the gene cluster. Above the genes, a
577 proposed pathway is depicted starting with cholesterol. The initial multiple steps are
578 indicated by the dotted arrow. After side chain cleavage, 17 β -hydroxy-5 α -androstane-3-
579 one (5 α -T) is formed as intermediate. Hsd4A catalyzes the dehydrogenation of the
580 hydroxyl-group at C17 [19]. Then 5 α -androstane-3,17-dione (5 α -AD) (and 5 α -T) can be
581 used as substrate by KSTD3, which introduces an unsaturated bond at C1-C2. The
582 product 1-(5 α)-androstene-3,17-dione (1-(5 α)-AD) is a substrate for Δ 4 KSTD encoded

583 by *kst4D*. 1,4-Androstadiene-3,17-dione (ADD) is converted into propionyl-CoA and
584 pyruvate via multiple enzymatic steps indicated by the dotted arrow.

585

586 **Figure 3 Expression of KSTD enzymes in *E. coli* cell free extract**

587 Coomassie Blue staining of SDS-PAGE gel loaded with cell free extract (CFE, 50 µg of
588 protein) of *Escherichia coli* BL21(DE) strains induced with 0.1mM IPTG. Lane 1:
589 marker; Lane 2: CFE with pET15b; Lane 3: CFE with pET15b-KSTD1 (53 kDa); Lane 4:
590 CFE with pET15b-KSTD2 (60 kDa); Lane 5: CFE with pET15b-KSTD3 (61 kDa) and
591 lane 6: CFE with pET15-Rv3537 (60.6 kDa). Estimated content of KSTD1 enzyme in
592 CFE is 40-50%, for KSTD2 15-20% and for KSTD3 and Rv3537 around 5%.

593

594 **Figure 4 Phylogenetic tree showing different KSTD enzyme clusters**

595 Phylogenetic tree with different amino acid sequences of (putative) 3-ketosteroid
596 dehydrogenases of well-known cholesterol-degrading actinomycetes and the gram-
597 negative bacterium *Comamonas testosteroni*. A clear separation between $\Delta 1$ KSTD and
598 $\Delta 4$ KSTD enzymes is apparent. The $\Delta 1$ KSTD enzymes cluster in at least 4 different
599 groups: $\Delta 1$ type 1 orthologues of KSTD1 of *R. erythropolis* SQ1, $\Delta 1$ type 2 orthologues
600 of KSTD2 of *R. erythropolis* SQ1, $\Delta 1$ type 3 orthologues of KSTD3 of *R. erythropolis*
601 SQ1, and a yet unknown type of $\Delta 1$ KSTD (tentatively designated $\Delta 1$ type 4) sofar
602 without a homolog of *R. erythropolis* SQ1.

603 The $\Delta 4$ KSTD enzymes cluster in at least 3 different groups of which only one group
604 contains a $\Delta 4$ KSTD of *R. erythropolis* SQ1.

605 Abbreviations: KSTD, *R. erythropolis* SQ1; Tes, *C. testosteroni*; MAP, *M. avium subsp.*
606 *paratuberculosis* str. k10; MSMEG, *M. smegmatis*; Rv, *M. tuberculosis* H37Rv; nfa, *N.*
607 *farcinica* IFM10152; ro, *Rhodococcus* sp. RHA1; orf, *R. equi*; KsdDAs, *A. simplex*;
608 KsdDRr, *R. rhodochrous*

609

610 **Figure 5 Alignment of amino acid sequences of $\Delta 1$ KSTD enzymes and fumarate**
611 **reductase reveals four conserved regions**

612 Alignment of the four conserved regions (I-IV) present in all $\Delta 1$ KSTD enzymes and
613 fumarate reductase of *Shewanella putrefaciens* MR-1 (Frd, accession number 1D4D_A).
614 KSTD1, KSTD2 and KSTD3 indicate the three different $\Delta 1$ KSTD enzymes of
615 *Rhodococcus erythropolis*, KsdD As represents KsdD of *Arthrobacter simplex*, and KsdD
616 Rr represents the KsdD of *Rhodococcus rhodochrous*. See experimental section for
617 accession numbers. Active center residues and residues involved in coordination of a
618 sodium ion in Frd are indicated by #. The cysteine residue characteristic for KSTD2
619 enzymes is underlined. The conserved threonine residue of $\Delta 1$ KSTD enzymes suggested
620 to be involved in substrate binding or catalysis is in capital and bold.

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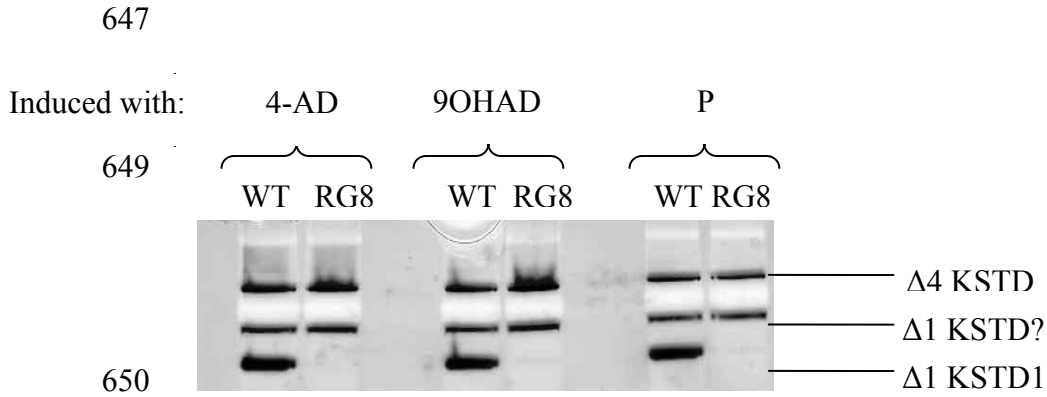
629

630 **Table 1 Isolated gene cluster of *Rhodococcus erythropolis* SQ1 and their**
 631 **homologues**
 632 Homologues of the 6 Open Reading Frames (ORF) of *R. erythropolis* SQ1 located on
 633 pJK2 and the (putative) functions of the proteins encoded.

Gene	Annotation	Annotated best hit (% identity)	Reference
<i>kst4D</i>	3-ketosteroid-delta4(5alpha)-dehydrogenase	TesI of <i>Comamonas testosteroni</i> (45%)	[12]
<i>kstD3</i>	3-ketosteroid-delta-1-dehydrogenase	KSTD (ro04532) of <i>Rhodococcus</i> sp. RHA1 (78%)	[19]
<i>hsaG</i>	Acetaldehyde dehydrogenase	HsaG (ro04534) of <i>Rhodococcus</i> sp. RHA1 (96%)	[19]
<i>hsaE</i>	2-hydroxypentadienoate hydratase	HsaE (ro04533) of <i>Rhodococcus</i> sp. RHA1 (87%)	[19]
<i>choG</i>	Cholesterol oxidase	BCO2 of <i>Brevibacterium sterolicum</i> (72%)	[27]
<i>hsd4B</i>	2-Enoyl acyl-CoA hydratase	HSD4B (ro04531) of <i>Rhodococcus</i> sp. RHA1 (83%)	[19]

634 **Table 2 Substrate profiles of KSTD1, KSTD2 and KSTD3 of *R. erythropolis* SQ1,**
 635 **and KSTD3 of *M. tuberculosis* H37Rv, expressed in cell-free extracts of *E. coli***
 636 Substrate profiles of KSTD1, KSTD2 and KSTD3 of *R. erythropolis* SQ1, and KSTD3
 637 of *M. tuberculosis* H37Rv, expressed in cell-free extracts of *E. coli* BI21(DE3)
 638 determined under standard assay conditions (see experimental procedures). Enzyme
 639 activities (V_{\max}) were calculated in $\text{nmol}^{-1}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ total protein in CFE and are
 640 expressed as percentage compared to activity with 5 α -androstane-3,17-dione which was
 641 set at 100% (2328, 860, 216, 178 are the 100% values of KSTD1, KSTD2, KSTD3_{SQ1}
 642 and KSTD3_{H37Rv}, respectively). K_{Mapp} indicates the apparent affinity constants in μM .
 643 Values shown are \pm SEM (n=3). n.d.: enzyme activity was below detection limits. Note
 644 that the low solubility of steroids may affect measurements, therefore the kinetic
 645 parameters do not represent absolute but relative values, providing a strong indication of
 646 the substrate preference of the KSTD enzymes.

Substrate	KSTD1		KSTD2		KSTD3 _{SQ1}		KSTD3 _{H37Rv}	
	Rel. V_{\max}	K_{Mapp}	Rel. V_{\max}	K_{Mapp}	Rel. V_{\max}	K_{Mapp}	Rel. V_{\max}	K_{Mapp}
17 β -hydroxy-5 α -androstane-3-one (5 α -T)	84% \pm 3	36 \pm 4	59% \pm 3	6 \pm 1	97% \pm 10	36 \pm 3	73% \pm 14	33 \pm 4
5 α -androstane-3,17-dione (5 α -AD)	100%	59 \pm 3	100%	13 \pm 3	100%	70 \pm 12	100%	166 \pm 16
5 β -androstane-3,17-dione	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-androstene-3,17-dione (4-AD)	574% \pm 14	104 \pm 5	249% \pm 7	5 \pm 1	n.d.	n.d.	n.d.	n.d.
9 α -hydroxy-4-androstene-3,17-dione (9OHAD)	655% \pm 30	119 \pm 11	186% \pm 8	58 \pm 9	n.d.	n.d.	n.d.	n.d.
1-(5 α)-androstene-3,17-dione (1-(5 α)-AD)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5 α -androstane-3 β -ol, 17-one	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1,4-androstadiene-3,17-dione (ADD)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone (P)	340% \pm 8	18 \pm 2	460% \pm 6	3 \pm 1	25% \pm 3	29 \pm 6	35% \pm 6	65 \pm 4
5 α -pregnan-3,20-dione (5 α -P)	32% \pm 2	21 \pm 5	12% \pm 1	7 \pm 1	47% \pm 8	14 \pm 4	n.d.	n.d.
23,24, bisnor 5 α -cholestane-3-one acid	69% \pm 2	74 \pm 4	60% \pm 2	26 \pm 4	n.d.	n.d.	n.d.	n.d.
11 α -hydroxy-19-norandrost-4-ene-3,17-dione	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11 β -hydrocortisone	88% \pm 14	557 \pm 120	70% \pm 2	64 \pm 5	n.d.	n.d.	n.d.	n.d.
4-cholesten-3-one	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5 α -cholestan-3-one	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dehydroisoandrosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.



651 **Figure 1**

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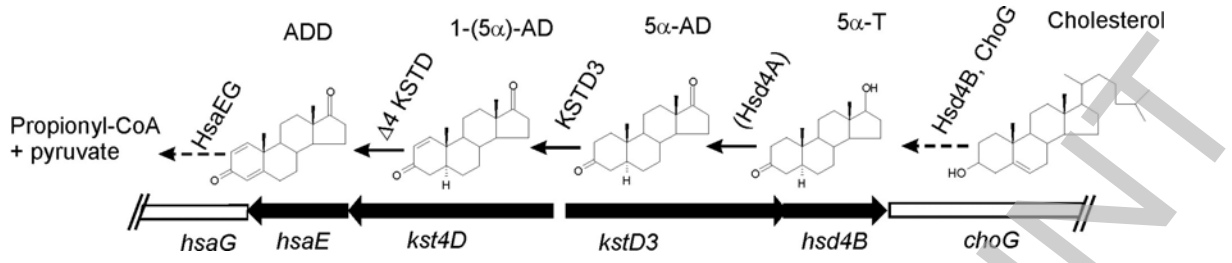
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Stage 2(a) POST-PRINT

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668 **Figure 2**

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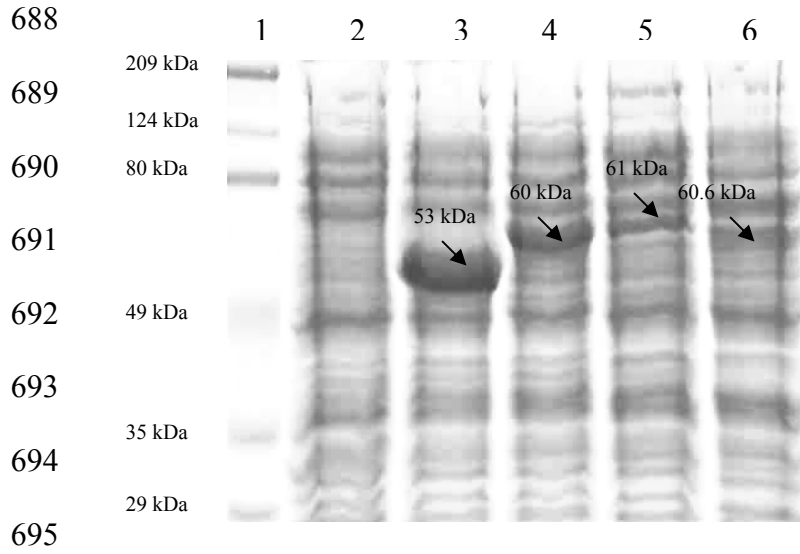
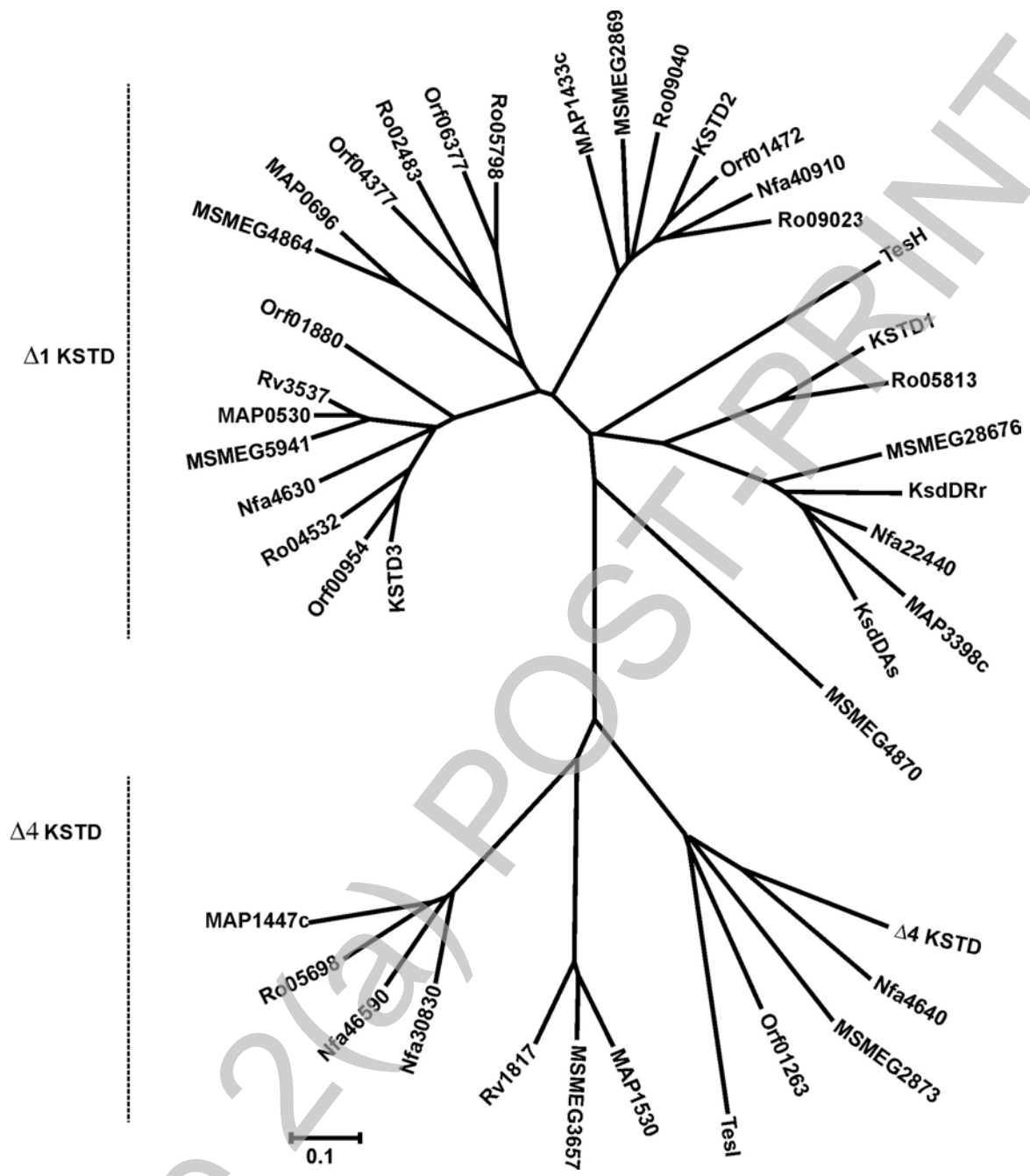


Figure 3

Stage 2(a) POST-PRINT

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Figure 4

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722			Region I	
723				
724	Frd	308	avviaaggfaknnervsky	
725	KsdD Rr	226	gvvlaaggfdqndemrgky	
726	KsdD As	227	gvllaaggfeandelrky	
727	KSTD1	224	gvlmaaggiegnaemreqa	
728	KSTD2	270	gvvlaaggfdhnmewrhky	
729	KSTD3	258	gvvlsgggfehnkemreky	
730	Consensus		gv--a-ggf--n-----ky	
731				
732			Region II	
733				
734				#
735	Frd	384	gaivvnregnrfrmneitrd	
736	KsdD Rr	301	ggifvdqdggarftneyapyd	
737	KsdD As	302	ggifvdgagrrfvnesapyd	
738	KSTD1	300	gglvvsagerylneslpyd	
739	KSTD2	350	gsfivdqtgrrfvneatdym	
740	KSTD3	336	gcmvmvmsgkrfgneaapyv	
741	Consensus		g---v---g-rf-ne	
742				
743			Region III	
744				
745				
746	Frd	446	tieelakqidvpaaelaktvtayngfvksgkdaqfer	
747	KsdD Rr	377	tleeagqigvpaeslkatvarwhelaakgvdedfgr	
748	KsdD As	378	tlpelaaligvpadalvatvarfnelvadgydadfgr	
749	KSTD1	373	tleeaaktglpadalrstvekfnadaaklgvdeefhr	
750	KSTD2	427	spaelarkvglpedafaesfqkfneaaaagsdaefgr	
751	KSTD3	416	tiaelaektglpvasltetvdrfngfarngkdedfgr	
752	Consensus		t--ela--i-vp---l--t-----n-----g-d--f-r	
753				
754			Region IV	
755				
756			#####	# # # #
757	Frd	495	yaleiapavhhtmglvidtkaevksektgkpitglyaagevtggvhganrlg	
758	KsdD Rr	438	haaqfgisdldgTkgglrtdtvgrvl-dsegapipglyaagntmaapsgtvypg	
759	KsdD As	439	haaafgisdlgTtkgglrtdtsarvlta-dgtpigglyaagntmaapsgttypg	
760	KSTD1	438	yaarivlsdlgTkgglvtdvngrvl-radgsaidglyaagntsaslsgrfypg	
761	KSTD2	492	yavkmtlsdlgTcggvqadenarvlre-dgsvidglyaigntaanafghtypg	
762	KSTD3	480	yaikmvpdldgTkggictdvngalre-dgsvidglyaagnasapvmghyagp	
763	Consensus		ya-----t-gg---d---v-----g--i-glya-g-----g---g	
764				

765 **Figure 5**

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